MICRORESPIROMETER AND ASSOCIATED METHODS

BACKGROUND OF THE INVENTION

Cross-Reference to Related Application

This application claims priority from commonly owned provisional application Serial No. 60/249,771, filed November 17, 2000, "Carbon Dioxide Microrespirometer."

Field of the Invention

The present invention relates to a device and method for determining gas evolution rates in solids and liquids, and, more particularly, to such a device and method for determining carbon dioxide evolution rates in a sample.

Description of Related Art

Respiration is a common indicator of biological activity. Respirometry, the measurement of respiration rates, has been applied to a broad spectrum of applied and environmental microbiology, such as toxicity, with treatability, process control, and prediction of biological oxygen demand (BOD_5) in wastewater treatment, assessment of metal toxicity, living soil microbial biomass, and food quality.

Respiration rates can be measured either by rates of oxygen consumption or CO_2 evolution. Rapid oxygen consumption rate can be measured by using an oxygen probe or a quantitative electrolytic cell. Most oxygen respirometers, however, are applicable only

to liquid samples. Oxygen respirometers with an electrolytic cell can be used to determine respiration of solid or semisolid samples, but their sensitivity is compromised.

Sensitive and rapid CO_2 respirometers based on infrared (ir) detectors have been developed in the past three decades and can handle solid samples with high speed and sensitivity. Instrumental respirometers are technically complicated and expensive if accuracy and sensitivity are needed. Noninstrumental CO_2 respirometers operated by an alkaline trap and acid–base titration have been in existence for many years. They are simple but relatively slow, with a measurement time in days, and less sensitive, with a detection limit in mL CO_2/day. Sensitive and rapid determination of respiration rates is highly desirable in monitoring microbial activity in food and environmental samples. A desired sensitivity, for example, would comprise one in the microliter CO_2 per hour level, and a rapidity of determination within about an hour.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide a device and method for determining gas evolution rates rapidly and sensitively.

It is another object to provide such a device and method for determining CO_2 evolution rates directly.

It is an additional object to provide such a device and method for use with solid or liquid samples.

It is a further object to provide such a device and method having a modest cost.

It is also an object to provide such a device and method operable under laboratory or remote site conditions.

These and other objects are attained by the present invention, a first aspect of which is a method for measuring an evolution rate of a gas from a sample. The method comprises the steps of pre-incubating a sample in gas communication with a solution comprising an alkaline solution and a pH indicator and permitting the alkaline solution to absorb carbon dioxide formed by the sample in an enclosed headspace. After the CO_2 absorption/evolution steady state is attained, from a change in the pH indicator is determined a time increment at which a small increment of the alkaline solution is substantially consumed by the CO_2 evolved. A calculation is made of a carbon dioxide evolution rate from the time increment, the small increment volume and concentration of the alkaline solution.

Another aspect of the invention is a device for measuring an evolution rate of a gas from a sample. The device comprises a sample vial having an opening into an interior space for containing a sample therein. The device further comprises a reaction chamber having an opening adapted for mating with the sample vial opening and a solution-receiving opening for receiving a solution comprising an alkaline solution and a pH indicator. The reaction chamber is dimensioned for receiving a predetermined amount of the alkaline solution to absorb formed CO_2 from a sample within the headspace.

A further aspect of the invention is a system for measuring an evolution rate of a gas from a sample. The system comprises a respirometer device as described above and means for determining from a change in color in the pH indicator a time increment at which a small increment of the alkaline solution is substantially consumed by the CO_2 from the sample.

The features that characterize the invention, both as to organization and method of operation, together with further objects and advantages thereof, will be better understood from the following description used in conjunction with the accompanying drawing. It is to be expressly understood that the drawing is for the purpose of illustration and description and is not intended as a definition of the limits of the invention. These and other objects attained, and advantages offered, by the present invention will become more fully apparent as the description that now follows is read in conjunction with the accompanying drawing.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic illustration of a microrespirometer of the present invention.

FIG. 2 is a graph of CO_2 absorption versus shaking rate of the microrespirometer.

FIG. 3 is a graph of CO_2 absorption and concentration of an alkaline solution in the microrespirometer.

FIG. 4 is a graph of CO_2 absorption rate versus CO_2 concentration in the headspace of the microrespirometer.

FIG. 5 is a graph of the headspace CO_2 concentration, expressed as a percentage of the final steady-state headspace CO_2 concentration versus time of pre-incubation for a range of respiration rates.

FIG. 6 is a graph of CO_2 evolution rate determined by the microrespirometer versus that determined by an infrared analyzer

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

A description of the preferred embodiments of the present invention will now be presented with reference to FIGS. 1–6.

The basis of the system **10** and method of the present invention is to establish a carbon dioxide absorption/evolution steady state between an alkaline solution and a sample. After the steady state is attained, an indicator comprising, for example, phenolphthalein, is used to indicate the end point of a small increment of the alkaline solution being consumed by the CO_2 evolved.

The system **10** of the present invention comprises a microrespirometer device **11** (FIG. 1), which in turn comprises a substantially transparent reaction chamber **12** and sample vial **13**. The reaction chamber **12** comprises a small alkaline trap with a total headspace of 6–7 mL having a small septum hole **14**. The sample vial **13** size is variable, and exemplary sizes include 25, 30, 40, and 75 mL (e.g., Fisherbrand EPA bottles, Suwanee, GA). The reaction chamber **12** and sample vial **13** are coupled through a standard threaded screw **15** and septum liner **16** to form a closed headspace **17**.

An alkaline solution **21** is injectable, such as using a syringe **18**, into the reaction chamber **12** via the solution-receiving opening **18**, and a sample **19** is placeable in the sample vial **13**. The alkaline solution absorbs the CO_2 in the headspace **17**. The indicator in the alkaline solution changes color when the alkaline solution is "consumed" by CO_2. Preferably the microrespirometer **11** is shaken at a fixed rate (e.g., 240 rpm) on an orbital shaker **20** to enhance CO_2 absorption.

The alkaline solution of the present invention comprises a solution of NaOH, $BaCl_2$, and indicator, with an equal molar ratio of NaOH and $BaCl_2$ and 0.5 mL indicator solution, with 0.5% phenolphthalein in 50% ethanol solution, per 50 mL alkaline solution. $BaCl_2$ in the alkaline solution precipitates the absorbed CO_2, which ensures the stoichiometry of 2 moles of alkaline spent per mole of CO_2 absorbed:

$$CO_2 + 2NaOH + BaCl_2 = BaCO_3(s) + 2NaCl \tag{1}$$

$BaCl_2$ also sharpens the change of color at the end point when a very low level of respiration is being determined. The alkaline solution is stored in a septum-capped vial to prevent absorption of CO_2 from the air. The alkaline solution is transferred through, for example, a syringe **18**.

Optimal operating conditions for the system **10** were determined with a series of experiments. The effect of shaking on the CO_2 absorption of the microrespirometer **11** was investigated by coupling microrespirometers **11** with empty 25-mL sample vials **13** in a glove box having a known CO_2 concentration, as determined with an ir CO_2 analyzer.

A 0.2-mL portion of 0.002*M* alkaline solution was injected into each reaction chamber **12**. The microrespirometers **11** were shaken at fixed rates of 100, 150, 200, 250, and 300 rpm. The time required to consume the alkaline solution in each microrespirometer **11**, as indicated by the indicator color change, was recorded. Each test was repeated in triplicate, and the results are plotted in FIG. 2. The CO_2 absorption is shown to increase as the shaking rate is increased from 100 to 250 rpm. The increase in CO_2 absorption levels off when the shaking rate exceeded 250 rpm. Shaking at 200 rpm or higher improves reproducibility of CO_2 absorption. A fixed shaking rate between 200

and 250 rpm is recommended for the microrespirometer **11** because the benefit of shaking is achieved while the difficulty of operation at higher rates is avoided.

The effect of alkaline concentration on the absorption of CO_2 in a closed headspace **17** was investigated at 25°C. A 25-mL sample vial was connected to an ir analyzer so that the vial **13** and the ir detector formed a closed headspace **17** in which air circulated continuously. The 25-mL vial **13** was shaken at 240 rpm on an orbital shaker **20**. 1-mL portions of 0.2, 0.1, 0.01, and 0.001*M* were injected into the vial **13** through the solution-receiving opening **18** at the beginning of the experiment, and the concentration of CO_2 in the vial **13** was recorded periodically.

The experiment was repeated twice, and the results are plotted in FIG. 3, where each dot represents a single measurement. It can be seen that as the concentration of alkaline solution decreases from 0.2 to 0.01*M*, the CO_2 absorption rate decreases as well. The CO_2 absorption rate does not decrease further as the alkaline concentration is reduced from 0.01 to 0.001*M*. It is not believed possible to have complete absorption of CO_2 in the headspace **17** of the microrespirometer **11** in a matter of hours when the concentration of the alkaline solution is less than 0.01*M*. The concentration of the alkaline solution has to be much less than 0.01*M* in order to determine CO_2 evolution rate at a microliter per hour level. The microrespirometer **11** therefore does not work on the principle of complete CO_2 absorption, but on an absorption/evolution steady-state principle that will be discussed in the following.

An alkaline solution of less than 0.0005*M* is not sufficiently stable to be used in the microrespirometer **11** because the possibility of contamination from ambient CO_2 is too

large for such low alkalinity. Phenolphthalein is not stable in alkaline concentrations exceeding 0.01*M*; the deep pink color fades away by itself within 1 h. Therefore, a preferred alkaline concentration range suitable for the microrespirometer **11** is between 0.01 and 0.001*M*.

The relationship between CO_2 absorption rate and the CO_2 concentration in the headspace **17** of the microrespirometer **11** was also investigated. Microrespirometers **11** with a 75-mL sample vial **13** were coupled in a glove box of known CO_2 concentration. Increments of 0.1 mL 0.002*M* alkaline solution were injected into the reaction chamber **12**. The microrespirometers **11** were shaken at 240 rpm, and the time required to consume each increment of the alkaline solution was recorded. The consumption of each increment of the alkaline solution, for example, 0.2 μmol alkaline, or 0.1 μmol CO_2, represents a 29.7-ppm (v/v) reduction of CO_2 concentration in the 82-mL microrespirometer **11** at 25°C. Each treatment was performed in triplicate, and the results are plotted in FIG. 4, with each dot representing a single measurement.

In using the microrespirometer **11** of the present invention, a portion of solid or liquid sample **19** is placed in the sample vial **13**, and the vial **13** is coupled to the reaction chamber **12**. 0.8 mL alkaline solution of a desired concentration is injected into the reaction chamber **12** using a syringe **18**. The respirometer **11** is shaken at a fixed rate, for example, 240 rpm, for 30 min, which comprises the pre-incubation, pre-steady-state period, ensuring that the alkaline solution is not completely consumed during this time. If the alkaline solution is about to be consumed, more alkaline solution is injected into the reaction chamber **12**. After the 30-min pre-incubation, pre-steady-state period the shaker

20 is stopped, and the alkaline solution in the chamber **12** is withdrawn to leave 0.1–0.2 mL. The respirometer **11** is continued to be shaken until the alkaline solution changes to a faint pink color. The shaker **20** is stopped immediately, and 0.1 mL alkaline solution is injected, shaking is resumed, and the time required to consume the alkalinity is recorded.

In an alternate embodiment, all the alkaline solution in the chamber **12** is withdrawn, and a new 0.1 mL portion of alkaline solution is injected prior to resuming the shaking.

In either case, once the first indicator change has been recorded, increments of 0.1 mL alkaline solution are injected a predetermined number of further times, for example, twice more, and the time required to consume each increment is recorded.

The average of the times required to consume each 0.1-mL increment is used to calculate CO_2 evolution rate using the following formula:

$$\text{carbon dioxide evolution rate } (\mu\text{mol/h}) = (0.1 \times 10^3 \times M/2)/60t \qquad (2)$$

where M is the concentration of the alkaline solution in mol/L and t is the time required to consume the 0.1-mL increment in min. The CO_2 evolution rate can be expressed in microliters per hour by multiplying the molar volume of CO_2 at a specific temperature.

The relationship between the CO_2 absorption rate of a 0.002M alkaline solution and the concentration of the CO_2 in the headspace **17** is shown in FIG. 4. In general, the CO_2 absorption rate has a positive curve-linear relationship with the concentration of CO_2. The CO_2 absorption rate of the respirometer **11** at a given temperature and shaking rate reflects the CO_2 concentration in the headspace **17**, which may not be the CO_2 evolution rate of the sample. However, if a sample reaches steady state with the alkaline solution in the respirometer at a given temperature and shaking rate, the concentration of CO_2 in the

respirometer would eventually reach a constant value when the CO_2 absorption rate equals the CO_2 evolution rate. For example, if the starting CO_2 evolution rate of the sample **19** is 100 μL/h, the CO_2 concentration of the respirometer **11** is increased to about 660 ppm and remain there because a steady state between CO_2 absorption and evolution is established. If the CO_2 evolution rate of the sample is 20 μL/h, the CO_2 concentration of the respirometer **11** is decreased to about 150 ppm, where an absorption/evolution steady state is established. The CO_2 evolution rate of a sample **19**, therefore, can be determined by the CO_2 absorption rate of the microrespirometer **11** when a steady state is established. That is, after a sample reaches steady state with an alkaline solution in a microrespirometer **11** of the present invention, the CO_2 evolution rate can be determined by the time required to consume a small increment of the alkaline solution, as shown in Eq. (2).

The minimum time required for a sample **19** in the respirometer **11** to reach steady state is deduced from a computer simulation based on a relationship between the CO_2 absorption rate and the CO_2 concentration of the respirometer **11** and the CO_2 evolution rate of the sample **19**. That is, the concentration of CO_2 in the headspace **17** after being shaken for a small increment of time Δt is

$$C_{i+\Delta t} = C_i + (E - A_{Ci})\,\Delta t / V_{\text{headspace}} \qquad (3)$$

where C_i and $C_{i+\Delta t}$ are the CO_2 concentrations of the respirometer at time i and time $i + \Delta t$, respectively. A_{Ci} is the CO_2 absorption rate of the respirometer at time i and is a function of the CO_2 concentration C_i. E is the CO_2 evolution rate of the sample **19**, and $V_{\text{headspace}}$ is the volume of the headspace **17**.

The mathematical relationship of A_{Ci} and C_i was generated by a nonlinear regression curve fitting program (Table Curve, Jandel Scientific, San Rafael, CA) using the data of FIG. 4. The regression enabled the calculation of A_{Ci} based on C_i. The values of A_{Ci}, C_i, and $C_{i+\Delta t}$ for each small time increment (0.5 min) of Δt were calculated and tabulated using a spreadsheet software (Excel, Microsoft, Redmond, WA) based on Eq. (3). Steady state is attained in the simulation when the CO_2 concentration in the respirometer approaches a constant, i.e., $(E - A_{Ci})$ approaches 0 and $C_{i+\Delta t}$ approaches C_i. The minimum time required to attain steady state is the sum of all small time increments, Δt, during which CO_2 concentration approaches a constant. The headspace CO_2 concentration, expressed as a percentage of the final steady-state headspace CO_2 concentration versus time of pre-incubation is presented in FIG. 5 for a range of respiration rates. Two headspace volumes of the respirometer, i.e., 12 mL (5 mL remaining headspace in the sample vial plus 7 mL in the reaction chamber) and 27 mL (20 mL remaining headspace in the sample vial plus 7 mL in the reaction chamber) were simulated in FIG. 5.

The results indicate that the smaller the headspace **17**, the quicker steady state is reached, and that the greater the CO_2 evolution rates, the quicker steady state is reached. For example, in the 12-mL headspace case, a 30 min pre-incubation, pre-steady-state period is sufficient for the measurement of all CO_2 evolution rates ≥ 1 μL/h. In the 27 mL headspace case, 100–107% of the steady-state value can be attained within 45 min for all CO_2 evolution rates, except the 1 μL/h case. The working range of the respirometer is designed to be 1–300 μL/h, which requires 30–45 min of pre-incubation time, according to the condition of this study, to measure accurately the CO_2 evolution rate. If the CO_2

evolution rate is very low (≤5 µL/h), the headspace **17** of the respirometer **11** should be kept minimal to hasten the reaching of steady state The respirometer **11** was designed so that the size of the reaction chamber **12** stays the same while the size of the sample vial **13** may vary according to the need of samples and the requirement of a minimal headspace **17**.

A validation experiment was performed by comparing results using the microrespirometer **11** with a method using an ir analyzer such as known in the art (FIG. 5). Portions of soil samples of relatively low CO_2 evolution rates (2–5 µL/h/g), unfrozen processed meat samples of medium CO_2 evolution rates (10–100 µL/h/5 g), and room-temperature milk samples of high CO_2 evolution rates (80–280 µL/h/20 mL) were placed in 25-mL sample vials **13**. The CO_2 evolved by microorganisms associated with each sample was determined by the microrespirometer **11** method of the present invention. A duplicate sample in another 25-mL sample vial **12** was also placed in a 250-mL flask, and the CO_2 evolution rate was determined by the ir analyzer method known in the art. The sample vials **12** in the microrespirometers **11** and those in the 250-mL flasks of the ir analysis method were exchanged, and the CO_2 evolution rates determined again with the alternate methods.

One of the advantages of the microrespirometer **11** is its ability to determine the CO_2 evolution rate accurately at the µL/h level in a short time. Determination of the CO_2 evolution rates at a µL/h level is quite a challenge even for a sophisticated ir method. The IR analyzer must be able to detect less than 10 ppm (v/v) changes of CO_2 concentration with certainty during a period of hours. The accuracy of an IR analyzer method is further

limited by the uncertainty of the volume occupied by a solid sample, and, therefore, that of the headspace, in most cases. Variation of headspace humidity, pressure, and temperature all affect the accuracy and precision of an ir respirometer. Because the microrespirometer method is based on the principle of CO_2 absorption-evolution steady state, its accuracy is not affected by headspace volume, humidity, pressure, or initial CO_2 concentration. The simplicity, noninstrumental nature, and very modest costs of the microrespirometer **11** make it available to many laboratory and field applications where accurate and rapid determination of respiration rate is desired.

In the foregoing description, certain terms have been used for brevity, clarity, and understanding, but no unnecessary limitations are to be implied therefrom beyond the requirements of the prior art, because such words are used for description purposes herein and are intended to be broadly construed. Moreover, the embodiments of the apparatus illustrated and described herein are by way of example, and the scope of the invention is not limited to the exact details of construction.

Having now described the invention, the construction, the operation and use of preferred embodiment thereof, and the advantageous new and useful results obtained thereby, the new and useful constructions, and reasonable mechanical equivalents thereof obvious to those skilled in the art, are set forth in the appended claims.

ABSTRACT

A method for measuring an evolution rate of a gas from a sample includes pre-incubating a sample with an alkaline solution and a pH indicator and permitting the alkaline solution to absorb formed carbon dioxide in an enclosed headspace. From the pH indicator at steady state is determined a time increment at which an increment of the alkaline solution is consumed by the CO_2. Carbon dioxide evolution rate is calculated from the time increment, the volume increment, and the alkaline solution concentration. A device for performing this measurement includes a sample vial and a reaction chamber having an opening adapted for mating with a sample vial opening and an opening for receiving the solution. The reaction chamber is dimensioned for pre-incubating the sample with the alkaline solution and for determining the time increment required for an increment of the alkaline solution to be consumed by CO_2.